REACTION TO INJURY IN THE OYSTER (CRASSOSTREA VIRGINICA)

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The comparative approach to pathology, which uses both cold-blooded vertebrates and invertebrates to advantage, was pioneered brilliantly by Metchnikov (1891) and has since been continued, somewhat sporadically, in France (Cantacuzène, 1923) and elsewhere (Cameron, 1932; Schlumberger, 1952). There still remains a tremendous dearth of information concerning the reaction of various invertebrates to injury and infection. A partial exception to this is the study of insect pathology (Steinhaus, 1949).

In a continuing study of pathological processes in the oyster we have found that the initial phase of phagocytosis of bacteria by the oyster amebocyte is often preceded by adhesion of the bacteria to the amebocyte so that the cell surface is literally covered with bacteria, and the sticking may be limited to the contact of the flagellum of the organism with the amebocyte so that the still motile bacterium becomes anchored. Secondly, the classical cellular clot formed by the agglutination of these amebocytes may be accompanied by an extracellular clot which immobilizes bacteria. And finally, the cellular clot may be directly observed within the vascular system of the living oyster and may be produced by the injection of an extract of oyster tissue.

Material and Methods

Most of the experiments were done on a so-called half shell preparation in which, after an edge of shell was knocked off, the shell was pried open with a knife until the adductor muscle was seen; then, with as little trauma as possible, the adductor muscle was cut and the upper shell removed. In good preparations this meant that a portion of the mantle and the muscle was cut, and the pericardium was left intact. Such preparations (Fig. 1) were kept in running sea water and used during the next several days. Some of these lived as long as a week or ten days, but had by that time gradually deteriorated, showing a loss of leucocytes from the blood and progressive infection and disintegration of the muscle. Heart blood was readily obtained from them at any time, and direct examination of the various vessels of the mantle, palps and gills was satisfactory under a Zeiss dissecting microscope (40 ×). Intracardiac injections were usually done directly into the ventricle, and blood was withdrawn from the auricle.

During these operations, the animals must obviously be damaged to a greater degree than were Stauber's preparations (1950) in which a window was made directly over the heart. However, they allowed direct examination of the entire gill and vascular system, and were used only as acute preparations. A limited

number of observations were made on oysters in which a hole was carefully drilled near the pericardium and the shell was then picked away until the sheath was exposed.

Observations on phagocytosis were made with a Zeiss phase microscope both at 500 × and 1250 ×. A drop of freshly obtained blood was placed on a slide, then either a drop of bacterial suspensions from a freshly grown culture of marine bacteria was added to it, or a small portion of the colony itself was added with a loop directly to the drop of blood. The preparation was covered with a #1 cover-

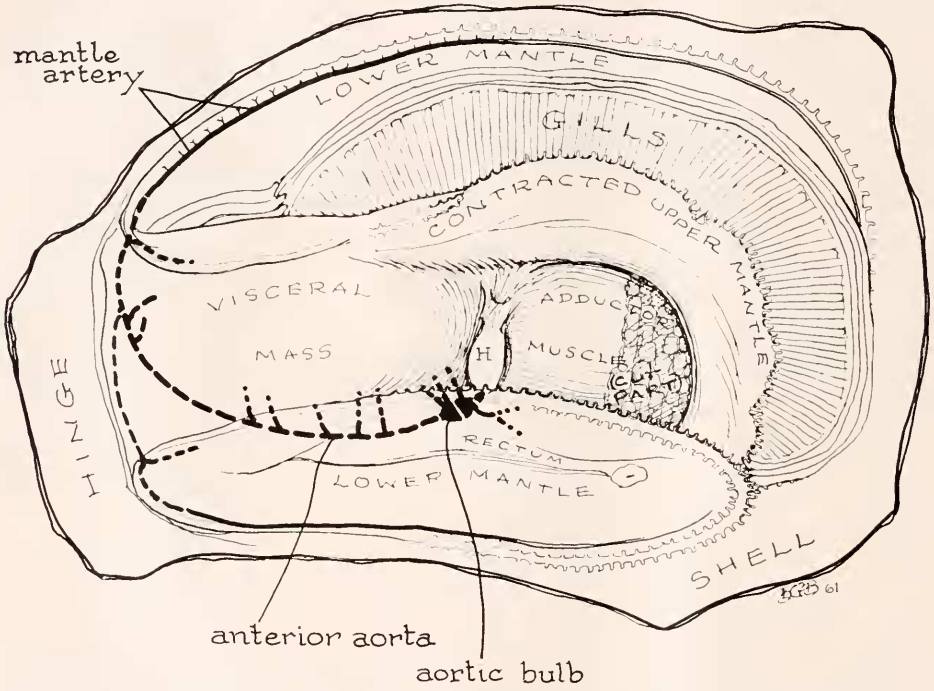


FIGURE 1. Diagram of half-shell preparation of oyster. Circulatory system indicated in heavy black line. Injections were made directly into the heart (H). Observations were made principally on mantle arteries.

slip, and if observations were to be continued, the entire preparation was ringed with Vaseline to prevent evaporation. Amebocytes remained viable for 12 hours or more under these conditions.

RESULTS

Phagocytosis

The amebocyte, which has been extensively studied in this and other molluscs (Fauré-Fremiet, 1927), is a granular round cell floating freely in the blood stream. On contact with glass it flattens out and moves continually over the surface of the slide. This motion under phase may clearly be seen to begin by the extrusion of a

series of filamentous pseudopodia which may be resolved with high power phase microscopy (Fig. 2) and which is shown in the accompanying electron microscope pictures. The spread of ectoplasm, illustrated in the accompanying figures (Figs. 3, 4, 5), then flows afterward, filling up the spaces between. Granules and other portions of the cell then flow into this region. A variety of cell forms may be observed on these slides; some of them lack granules, others contain large wavy frills of pseudopodia, other large amorphous but refractile inclusions. Since the amebocyte may both lose its granules and may ingest large amounts of material, we are unable to say whether these represent different types of cell or physiological variants of one type. Most of the cells observed during the process of phagocytosis were granular cells.

One of the most remarkable facts which was observed early in the study was the absence of phagocytosis. Frequently an amebocyte was seen to approach a bacterium with its fibrous processes, then either to reverse its flow or turn aside. During the course of several hours this behavior was repeated continuously and no phagocytosis was observed. Since it is so obviously contrary to established ideas of the importance of phagocytosis, and specific studies on phagocystosis of food particles by the oyster, the observations were repeated with a number of bacteria, and it was found that excellent phagocytosis might be obtained with a certain bacterium, yet little if any phagocytosis was observed in amebocytes from the same oyster if another preparation of bacteria was introduced. Repeated attempts were made to determine whether such failure of phagocytosis was due to the strain of bacteria, or to a combination of certain bacteria with amebocytes from certain oysters. It was not possible from day to day to find a combination of amebocytes and bacteria which did not phagocytize, but no observations were repeated within a few hours of each other and it remains likely that there is an undiscovered factor important in phagocytosis which is responsible for this variation.

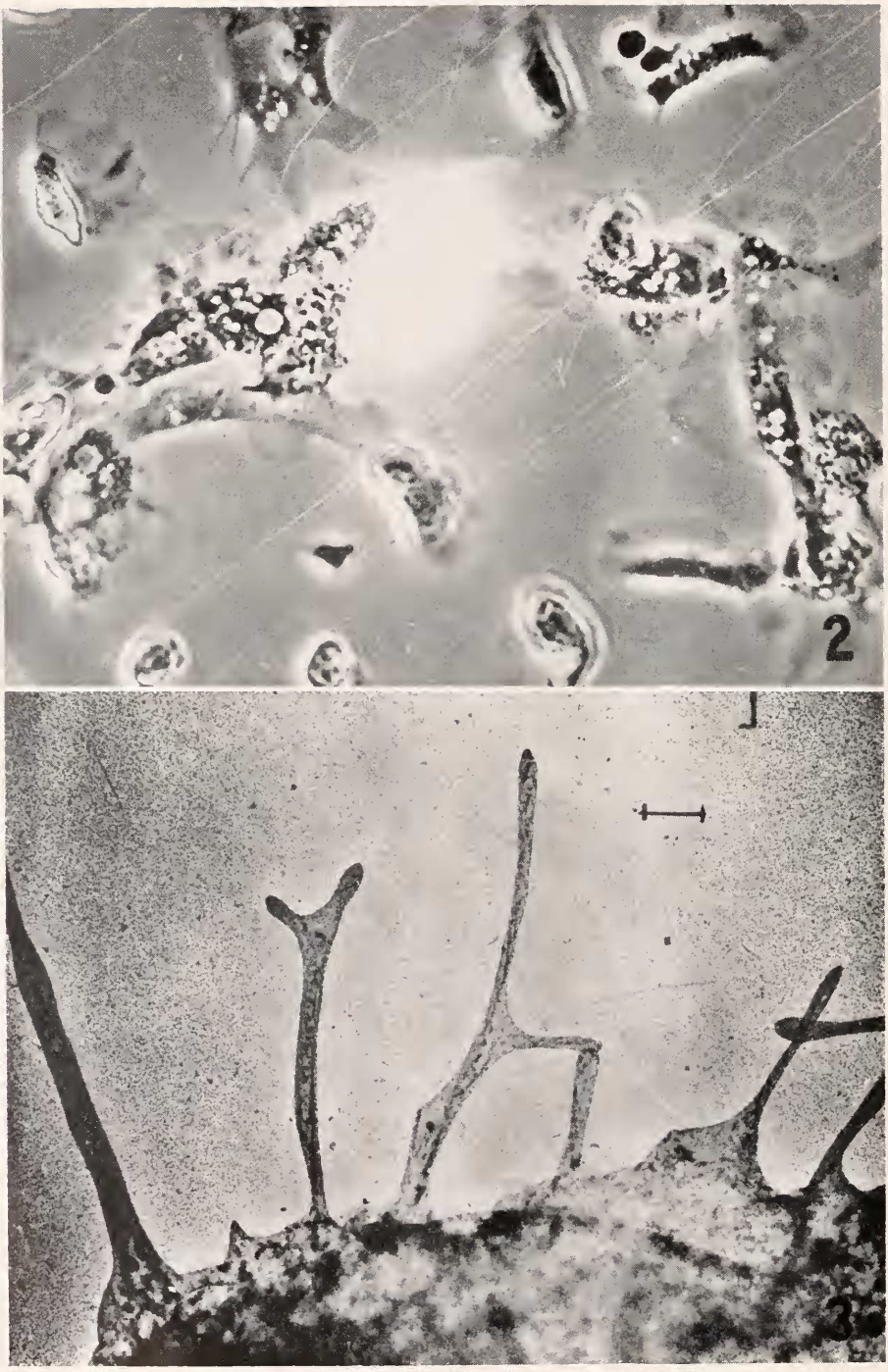
When a successful combination was obtained, and particularly when the bacterium used was motile, phagocytosis was usually preceded by a massive sticking of the bacteria to the amebocyte, so that the amebocyte resembled a porcupine. Ingestion followed this phase (Fig. 4). Some incidental observations on phagocytosis by amebocytes of the marine worm, *Urechis,* showed the adhesion of bacteria to be limited to the portion of the cell which had spread out on the glass.[1] In the oyster, however, since extrusions of the cell appeared from all sides it was not possible to determine whether all portions of the amebocyte were equally sticky.

The curious anchoring of motile bacteria to amebocytes which renders them unable to leave the amebocyte while they seem at the same time to have no contact with it, was explained by a fortunate electron microscope picture (Fig. 5). The presence of the unipolar flagellum wrapped around the filamentous pseudopodia fully explains the continual tugging and jerking at an invisible anchor.

Extracellular clot formation

During the summer of 1956, and in the two succeeding summers, the formation of a definite extracellular clot was observed (Fig. 6). It could be seen only under phase microscopy and seemed similar in texture and formation to a clot described

[1] These observations were made originally by Mr. Stuart Krassner, to whom we are indebted for permission to include this material.



FIGURES 2–3.

by Grégoire (1952) in a variety of insect bloods. The clot was found in only about one-third of the oysters which were examined, and was not present in the same oyster at all times. Furthermore, we have been unable to determine the conditions under which it may be consistently formed in an individual oyster. However, we were able to reproduce this extracellular clot throughout the summer months of the last three years, and have been clearly able to rule out artifacts of preparation.

The clot was first noticed in slides which had been kept for continued observation of phagocytosis. It tended to occur near the occasional small air bubble which was entrapped beneath the coverslip in the sealed slide. It was not present immediately but usually appeared in 15 minutes to half an hour, so that it was suspected of being a local drying phenomenon until it was seen in slides which had no appreciable air bubbles, and in sealed hanging drops and direct preparations. Attempts to relate it to the time of day, time since opening of the oyster, amount of trauma, and sex of oyster, failed. A mixture of pericardial fluid and blood did not affect the process. A higher percentage of extracellular clots seemed to be obtained from oysters which had been forced-starved by keeping them out of the sea water, and then replacing them; but this produced positive results in only about half of the cases. Since the clot had been originally observed in a preparation to which bacteria had been added, repeated comparisons were made in the presence and absence of the bacteria. In most cases, when the clot was obtained in the presence of the bacteria, it was also found in the control slide to which bacteria had not been added, though it was usually less extensive.

It seems to have a real rôle in the repair of traumatized tissue, for: 1) it was found already developing in small cellular clots taken directly from the heart; 2) it occurred predominantly around clumps of cells; 3) bacteria were immobilized by its development (Fig. 7); and, 4) it was obtained both immediately after opening an oyster and from some preparations which had been on the half shell as long as 24 hours. Its possible relation to cycles of feeding by the amebocytes is unknown.

Intravascular clots

There is a rapid clumping of cells when oyster blood is withdrawn in glass vessels, which is also the case with many other invertebrate bloods. Direct observation of traumatized blood vessels shows the formation of the same type of cellular clot at the open end of the vessel, so that within a few minutes of cutting, the clotted cells have effectively sealed the end. Similar clumps of amebocytes are observed directly covering the cut end of the adductor muscle in our preparations, and oysters which had been repeatedly bled develop a shaggy pericarditis which consists of masses of these clumped cells. However, in oysters which have had minimal trauma and have remained in clean running sea water for several hours after being opened, the circulation may be fully effective and direct examination of the distended vascular system was possible. In such a view, the cells are seen moving to and fro,

FIGURE 2. Phase micrograph of oyster amebocyte on glass, approximately 600 times.

FIGURE 3. Electron micrograph of whole cell preparation of amebocyte. Filamentous pseudopods extend away from the cell edge. This and succeeding electron micrographs were made by allowing the amebocytes to spread out on a collodion film. The cells were fixed with osmium vapor, washed and the film placed on grids; 9,000 times.

FIGURES 4–5.

and relatively few of them are clumped. When the circulation is sluggish they may be found lining the lower side of a vessel, but they readily move from one portion to another as the oyster is tilted. The obvious question whether a particular portion of the tissue was responsible for the formation of the cellular clot was tested by making a sea-water extract of gill tissue, centrifuging the extract and injecting about 0.1 cc. of the relatively clear supernatant directly into the heart. The material immediately spread throughout the animal and an interesting series of events set in. If the oyster had relatively large numbers of cells so that the blood was milky in appearance, the first reaction was the formation of large curd-like clumps of loosely aggregated cells. These became more dense, soon ceased to flow back and forth, and within 10 to 15 minutes were stuck in tight clumps to the edge of the vessel, and the fluid itself appeared perfectly clear. The vessel frequently decreased in size, particularly if the heart happened to cease beating. In many cases, some flow back and forth in the mantle vessels continued even though there was no visible heart beat, presumably from the action of the accessory heart (Fig. 1). Within about two hours after the injection, most of the effects had worn off: the heart was beating, the blood was again flowing freely, and relatively few clumps were seen. Individual cells were observed flowing freely in the large vessels or moving in and out of the fine branches of the palps or the gills. When these oysters were reinjected with the original extract, an apparently full-fledged repetition of the reaction was observed.

The reaction was not obtained by the injection of sea water, of suspensions of carmine, or of bacteria of several sorts, though a moderate "curdling" of the blood was seen after the injection of heavy suspensions of bacteria.

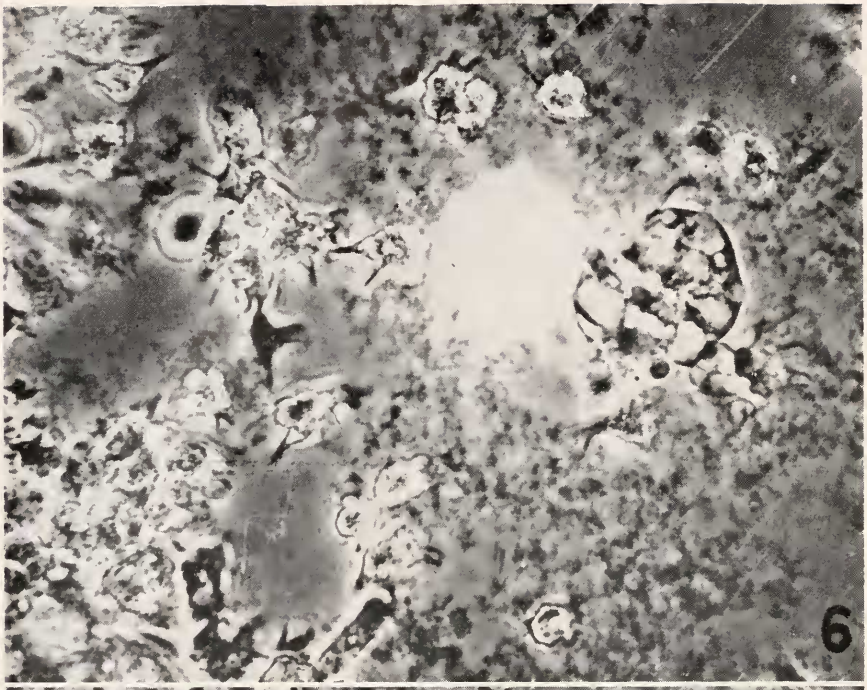
India ink of two sorts was then injected in suspensions of sea water. The usual preparation of colloidal ink when injected caused prompt clumping of cells, a cessation of heart beat, and the probable development of intravascular clumps like those seen following the injection of tissue extracts. However, the black masses of material which were partially phagocytized, as described by Stauber (1950), obscured the observation. A preparation of "Pelican" India ink, which lacks the gum coating present in most commercial India inks, produced a much milder reaction (Muller, 1927a, 1927b). The particles were soon phagocytized as small particles or clumps without major changes in the circulation itself, just as carmine particles had been.

Discussion

The capacity to react to injury, an essential function of living cells, is basic to studies in pathology. Following Metchnikov (1891), who began with a marine echinoderm embryo, the greatest attention of pathologists when studying invertebrates has concentrated on the wandering cells or amebocytes. From a comparative pathological point of view, at least three phenomena are contained in the oyster in this one cell. These are phagocytosis of invading bacteria, inflammation, and thrombosis. Since the amebocyte is the only circulating cell of the blood in the oyster, and since cellular clots are the common mechanism of closing gaps in the

Figure 4. Beginning phagocytosis of uniflagellate bacterium. The flagellum is coiled around several pseudopods of the amebocyte; 14,000 times.

Figure 5. An electron micrograph showing later stage in phagocytosis of bacterium; 9000 times.



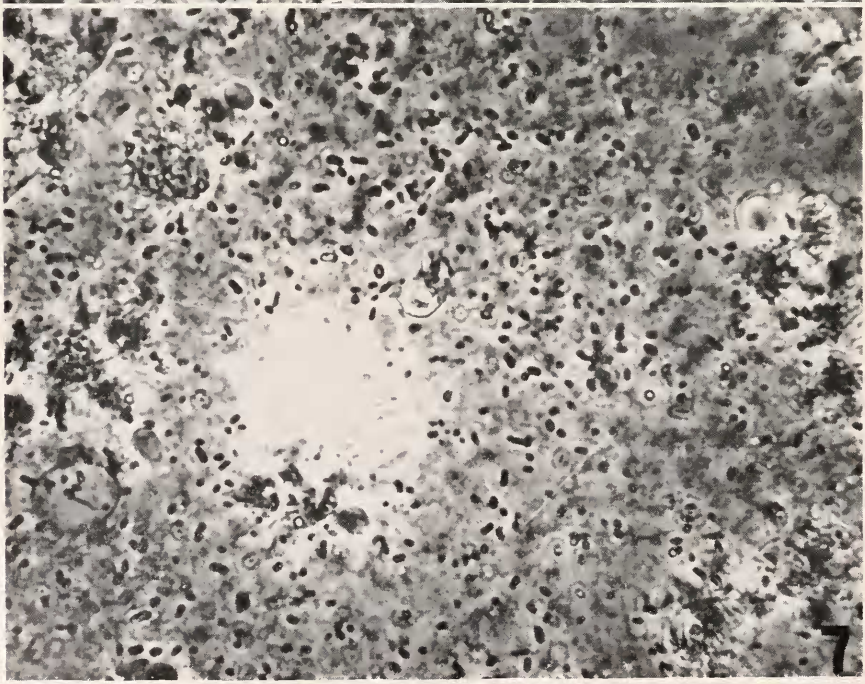


FIGURE 6. Phase micrograph showing extracellular clotting of amebocytes; 1000 times.
FIGURE 7. Similar extracellular clot with bacteria involved in clot; 1000 times.

vascular system among invertebrates (Geddes, 1880; Cuénot, 1891), it is of course impossible to separate the function of inflammation whereby a white cell in vertebrates becomes adherent to a vessel wall and migrates through it, and that of the adherence of many amebocytes together to form a tight clump which blocks the free flow of blood.

An ideal invertebrate in which to follow the above processes would allow direct observation of the vascular channels without trauma, and would from the bacteriological point of view allow for external sterilization and thus obtaining of blood without contamination by the surrounding fluid or air. In this regard the oyster and other molluscs have no external surface which may be sterilized and then punctured, and have no extension of the vascular system that may be observed without the introduction of trauma. Thus, though a variety of studies have been done on diseased oysters (Herdman and Boyce, 1899; Roughley, 1926; Stauber, 1945; Mackin, 1951; Mackin, *et al.,* 1952), there is little direct information on the pathogenesis of any of the disease states.

Phagocytosis of food material for transport through the oyster, and of particulate matter has been studied rather extensively (Yonge, 1926; Takatsuki, 1934), primarily by following the events in sequence by histological sections.

Phagocytosis itself was first observed about a hundred years ago, by Haeckel (1862), who injected particulate dyes into molluscs so that the distribution of the vascular system might be determined. He pointed to the potential importance of the phenomenon in nutrition. Then came the disclosure by Metchnikov of the role of phagocytosis as a defense mechanism (1884, 1891). In the oyster and other molluscs the importance of the amebocyte in digestion, transfer of food, and repair, has been firmly established (Yonge, 1928; Wagge, 1955). Recently Tripp (1960), has followed the fate of several species of bacteria in oyster tissue following intracardiac injection of large numbers. He has shown that phagocytosis may be apparent in the cells circulating within the vessels and subsequently in the tissues. Intracellular digestion appeared to be a major mechanism of disposal of the bacteria. In several infectious diseases of the oyster the presumptive agent is thought to be disseminated by the amebocyte (Orton, 1923). It was therefore a surprise to us to find that there was a marked variation in the phagocytosis of different preparations of bacteria by leucocytes of the same oyster. Attempts to show increased phagocytosis in the presence of mucus from the gill, of disintegrating crystalline style material, or of extracts of the hepato-pancreas failed.

Although "surface phagocytosis" (Wood, 1951–1952) took place in the process of the flow of amebocytic protoplasm around bacterium, it was not always the explanation, for masses of bacteria were found stuck to the surface of amebocytes in most of the cases where phagocytosis was apparent. The direct adherence of the amebocyte to the bacterium itself was highlighted by the observation of the flagellar adherence of the bacteria to the amebocyte so that it was unable to escape from the amebocyte.

The evolutionary need for extracellular clot formation becomes greater when the amebocytes or leucocytes have much less direct contact with each other because of the presence of large numbers of red cells. However, extracellular clot or gel formation is well developed in several invertebrates (Grégoire and Florkin, 1950; Loeb, 1910; Grégoire, 1952; Bang, 1956) in which the predominant circulating

cells are directly involved in clot formation (Yonge, 1926). The presence of this extracellular gel, which seemed fully able to limit bacterial motion in many of the oysters which we examined, may indicate that additional advantage is to be gained from such mechanisms of thrombosis which extend beyond the cell. The possible role of this extracellular material in rendering bacteria more susceptible to phagocytosis needs further study. The origin of this extracellular gel from the extrusion of the many cellular granules is an obvious possibility which has not been investigated.

Direct observations of the formation of the cellular clot at a point of traumatic rupture of a vessel, the accumulation of great numbers of these cells on the heart when it is exposed to sea water by opening the pericardium, and the accumulation of amebocytes at the cut edge of the adductor muscle, led to the question as to the effect of tissue extracts. It was soon found that a fresh crude sea-water extract of ground gill tissue, when injected directly into the heart, caused a rapid clumping of cells and the tight adherence of these cells to the vessel wall, so that the circulation was greatly slowed or stopped. Injection of sea water, of bacterial suspensions, and of carmine, failed to cause similar marked effects. Thrombosis accompanied by phagocytosis was rapidly produced by the injection of certain preparations of

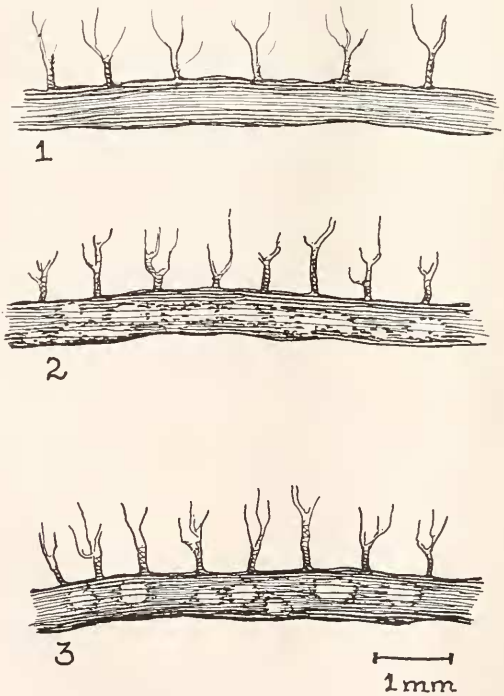


FIGURE 8. Diagram of observations of mantle artery: (1) shows the clear appearance of the vessel under normal conditions. Individual amebocytes may be seen poorly and are not indicated here. (2) Beginning clumping amebocytes within the vessel. They are loosely clumped and move rather freely in the vessel. (3) Amebocyte clumps which have contracted into tight balls of thrombus and are adherent to the vessel wall.

India ink, but not by a preparation which is stated to lack the shellac coating which in itself causes extensive thrombosis.

Our experiments have been limited to acute short term experiments. Several other molluscs have been used in the study of chronic processes (Drew and de Morgan, 1910; Zawarzin, 1927), and the importance of epithelial tissues, mucous sheets and chronic fibrous tissue "repair" needs extensive exploration (Kedrowsky, 1925; Labbe, 1929).

Summary

1. *In vitro* phagocytosis of marine bacteria by fresh oyster leucocytes, though readily demonstrable in most cases, was by no means an invariable phenomenon. When it occurred, it was frequently accompanied by a massive sticking of bacteria to the leucocytes. The flagellar portion of the bacterium might be so caught by the amebocyte that the bacterium was unable to escape, even though the body was not in contact with the amebocyte.

2. An irregular but repeated formation of an extracellular clot is described as seen *in vitro* by phase microscopy. Reasons for believing that it is a true phenomenon in the oyster are given.

3. Intravascular clotting or thrombosis was produced by the intracardiac injection of tissue extracts. The clotting disappeared spontaneously within two hours after the injection.

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